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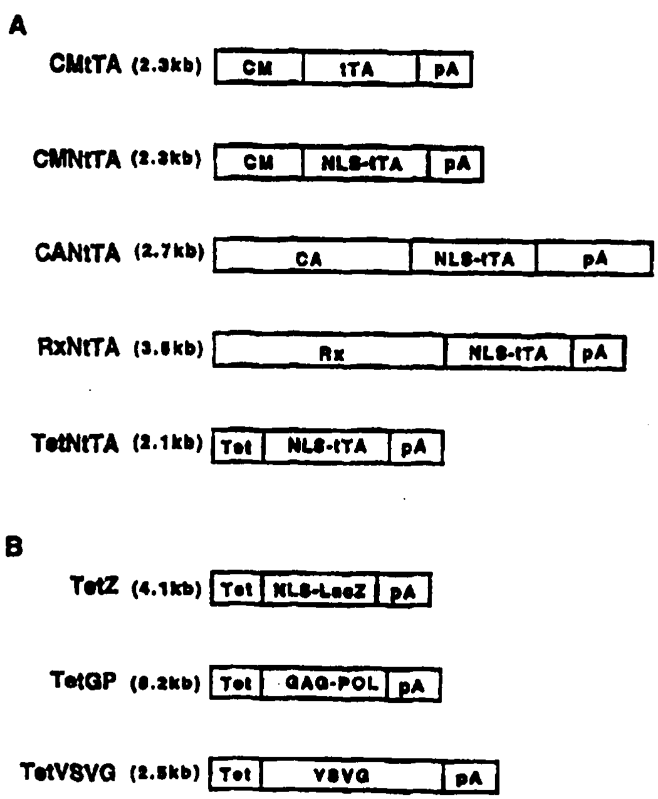
(57) Abstract

Recombinant genes having integrated thereinto a nuclear localization signal (NLS) gene and the tetracycline activator (tTA) gene, as well as viruses containing these recombinant genes and animal cells infected with these viruses.



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DESCRIPTION

TETRACYCLINE TRANSACTIVATOR HAVING A NUCLEAR LOCALIZATION SIGNAL JOINED THERETO

Technical Field

This invention relates to a system for the expression of genes in animal cells. More particularly, it relates to recombinant genes which can control the expression of the introduced gene strictly, and their uses.

Background Art

In many cases, substances other than the components indispensable for the growth of cells are inductively synthesized as required. Consequently, if an exogenous gene is introduced into animal cells for the purpose of causing the cells to produce a certain protein, the protein synthesized by the constant expression of the introduced gene may affect the growth of the cells into which the exogenous gene has been introduced, or may exhibit toxicity to the cells.

In order to solve these problems, there have been used inducible promoters derived from eucaryotic cells, such as the metallothionein gene (E.K. Mayo et al., Cell, Vol. 29, pp. 99-108, 1982), heat shock genes [L. Nouer et al., "Heat Shock Response" (edited by L. Nouer), CRC Publishing Co.] and several hormone genes (F. Lee et al., Nature, Vol. 294, pp. 228-232, 1981). However, when these promoters derived from eucaryotic cells are used, the introduced gene is slightly expressed even under uninduced conditions to produce a small amount of protein. Moreover, it has also been reported that the inducible promoter of the lac gene of procaryotic cells functions even in animal cells (M. Hu et al., Cell, Vol. 28, pp. 555-566, 1987). However,

this promoter is also unsatisfactory for the purpose of controlling the production of the desired gene product strictly.

As a gene expression system derived from procaryotic cells, other than the lac gene, Bujard et al. have developed and reported a tetracycline repressor (tetR)/tetracycline operator (tetO) gene expression system using a gene (also referred to as the tTA gene) encoding the tetracycline-controlled tetracycline trans-activator (tTA). tTA combined with tetR cannot bind to tetO in the presence of tetracycline, so that no expression of the gene occurs. On the other hand, tTA can bind to tetO in the absence of tetracycline, resulting in the expression of the gene. Thus, the use of tTA serves to control the switching-on/off of the expression of a gene according to the absence or presence of tetracycline.

Under the existing circumstances, however, even if the expression of the introduced gene is suppressed by the addition of tetracycline, the expression of the introduced gene is observed to an extent of about 0.01% based on the level observed under unsuppressed conditions. That is, the expression cannot be suppressed completely (M. Gossen et al., Proceedings of National Academy of Science, USA, Vol. 89, pp. 5547-5551, 1992). Thus, a system which can strictly control the expression of genes introduced into animal cells has not been reported so far.

Meanwhile, in order to generate packaging cell lines capable of producing retroviral vectors, it has conventionally been necessary to introduce at least one of the gag, pol and env genes which are indispensable for the replication of retroviruses. In such cases, it may frequently happen that, according to the locations at which the vector genes are introduced into the cell chromosomes, their expression is not completely sup-

pressed where unnecessary ("leakiness") or the level of their expression is low where necessary. Consequently, after introduction of the genes, considerable labor is required to select an excellent packaging cell line. Moreover, when the envelope protein of a retrovirus is modified to change the infective specificity of the retrovirus, it has been necessary to generate vector genes into which the genes encoding the envelope proteins of individual viruses are separately integrated. Thus, the generation of individual vectors requires a very laborious procedure.

Accordingly, an object of the present invention is to provide a gene expression system in which the expression of the introduced gene is completely or substantially suppressed under uninduced conditions to exert no influence on the growth of the cells having the gene introduced thereinto, whereas the introduced gene can be expressed under induced conditions to produce the desired protein.

Another object of the present invention is to provide a means for permitting the easy construction of pseudotyped retroviral vector production systems which can replace conventional packaging cell systems.

Disclosure of Invention

As a result of extensive investigations, the present inventor has now found that the above objects can be accomplished by integrating an NLS gene serving as a nuclear localization signal into the tetracycline-controllable gene expression system developed by Bujard et al. In short, it has been found that a virus containing a gene which comprises a combination of an NLS gene and the gene encoding the tetracycline transactivator (tTA) (i.e., the tTA gene) and which also has a promoter gene integrated thereinto shows enhanced gene expression in the absence of tetracycline, whereas the

gene expression is almost completely suppressed in the presence of tetracycline.

Thus, according to the present invention, a recombinant gene comprising an NLS gene and the tTA gene is provided as a means for accomplishing the above objects. In a preferred embodiment for accomplishing the objects of the present invention, the aforesaid NLS and tTA genes are integrated in-frame, i.e., in such a way that the proteins obtained as their gene products exhibit the desired effects. No limitation is placed on the positions where these genes are integrated, so long as their gene products exhibit the desired effects.

Brief Description of Drawings

Fig. 1 schematically illustrates, in terms of gene transcriptional units, typical recombinant genes of the present invention in comparison with well-known genes. The abbreviations given in this figure have the following meanings,

CM: Cytomegalovirus promoter.
CA: Promoter comprising the cytomegalovirus enhancer joined to the β-action promoter.
Rx: Promoter comprising the cytomegalovirus enhancer joined to the Moloney murine leukemia virus promoter.
Tet: Tetracycline promoter.
tTA: Tetracycline transactivator.
NLS: Nuclear localization signal.
pA: poly(A) signal.

Fig. 2 is a graph showing the expression efficiency in an expression system using a recombinant gene of the present invention, in comparison with the expression efficiency in the conventional gene expression system developed by Bujard et al.

In this graph, the circles represent the results obtained with CMNtTA (inventive) and the squares

represent the results obtained with CMtTA (comparative). The open symbols represent the expression efficiencies observed in the absence of tetracycline, and the filled symbols represent the expression efficiencies observed in the presence of tetracycline.

Fig. 3 is a graph showing variations in gene expression efficiency according to the type of the promoter used in a gene expression system of the present invention.

In this graph, the circles, squares and triangles represent the expression efficiencies of the introduced gene observed when the CA, Rx and Tet promoters were used for NtTA, respectively. The open symbols represent the data obtained in the absence of tetracycline, and the filled symbols represent the data obtained in the presence of tetracycline.

Fig. 4 is a graph showing variations in gene expression efficiency according to the concentration of tetracycline, for several gene expression systems of the present invention (CMNtTA, inverted triangles; CANtTA, triangles turned sideways; TetNtTA, triangles; RxNtTA, squares) and conventional systems employed as comparative examples (TetZ alone, diamonds; CMtTA, circles).

Fig. 5 is a set of electron micrographs showing the morphology of cells (or organisms) having been subjected to β-Gal staining for detecting the expression of the lacZ gene in gene expression systems (or infected cells) employed as comparative examples. In this figure, a and b correspond to the system using TetZ alone (comparable), and c and d correspond to the system using CMtTA (comparative). In each system, the former (a, c) shows the cells cultured in the absence of tetracycline and the latter (b, d) shows the cells cultured in the presence of tetracycline.

Fig. 6 is a set of electron micrographs showing the morphology of cells (or organisms) having been

subjected to β-Gal staining for detecting the expression of the lacZ gene in gene expression systems (or infected cells) in accordance with the present invention. In this figure, e and f correspond to the CMNtTA system, g and h correspond to the CANtTA system, i and j correspond to the RxNtTA system, and k and l correspond to the TetNtTA system. In each system, the former (e, g, i, k) shows the cells cultured in the absence of tetracycline and the latter (f, h, j, l) shows the cells cultured in the presence of tetracycline.

Detailed Description of the Invention

The present invention also provides the aforesaid recombinant gene into which a promoter gene indispensable for the expression of those genes is also integrated. Usually, it is advisable to integrate the promoter so as to be located upstream of the aforesaid NLS and tTA genes. According to the present invention, any promoter can be used without regard to its origin and type, so long as it enables the expression of the aforesaid NLS and tTA genes. However, the NLS gene which can conveniently be used for the purposes of the present invention is one derived from the large T protein (or antigen) of SV40 virus, and the promoters which can conveniently be used include the CM promoter of cytomegalovirus, the Rx promoter comprising the enhancer of cytomegalovirus joined to the promoter of Moloney murine leukemia virus, the CA promoter comprising the enhancer of cytomegalovirus joined to the promoter of β-actin, and the Tet promoter of the tetracycline gene, though the present invention is not limited thereto.

According to the present invention, a gene encoding a foreign protein may further be integrated into the aforesaid recombinant gene. As will be described later in detail, the recombinant gene of the present invention is such that its expression can be

strictly controlled by the presence or absence of tetracycline. Consequently, even when the expression of any of the aforesaid genes included in the recombinant gene exerts an adverse influence on the growth of cells containing the recombinant gene, its expression can be suppressed almost completely. Moreover, its expression can also be enhanced in some cases.

Thus, there is provided an expression system suitable for use in the production of a particular protein for any intended purpose. Accordingly, the foreign protein contemplated in the present invention can be any protein according to the intended purpose. Specific examples of the foreign protein include, but are not limited to, viral envelope proteins, hormones, cytokines, and the receptors for hormones and cytokines.

The above-defined recombinant gene may conveniently be used in the form of a plasmid constructed by carrying the recombinant gene on a specific vector. Typical examples of the plasmid in accordance with the present invention are mentioned in the examples which will be given later, but all plasmids that will readily occur to those skilled in the art on reference to these typical examples are intended to be within the scope of the present invention.

Moreover, the present invention also provides viruses containing the aforesaid recombinant gene. For the purposes of the present invention, the preferred virus is one using an adenovirus as the host virus. As the adenovirus, there can conveniently be used both a mastadenovirus whose host is a mammal and an aviadenovirus whose host is a bird, but the former is preferred.

It is preferable that the NLS gene, the tTa gene and the foreign gene are integrated downstream of the promoter.

Furthermore, the present invention also provides animal cells transfected with the aforesaid vi-

ruses. Preferred animal cells are mammalian cells transfected with mastadenoviruses in accordance with the present invention. Specific examples of such mammalian cells include A375 cells (ATCC CRL-1619; a human melanoma cell strain), T98 cells (ATCC CRL-1690; a human cerebral tumor cell strain), A172 cells (ATCC CRL-1620), U373 cells (ATCC HTB-17) and murine 3T3 cells (ATCC CCL-163). By growing the cells thus obtained, the production of, for example, a foreign protein can be carried out efficiently.

More specifically, each of the above-described subjects of the present invention can be obtained according to the procedures described below or in the examples will be given later.

For example, a recombinant adenovirus is first generated by joining the tetracycline transactivator (tTA) gene to the downstream side of the promoter of cytomegalovirus (i.e., the CM promoter; see M. Gossen et al., Proceedings of National Academy of Science, USA, Vol. 89, pp. 5547-5551, 1992). On the other hand, another recombinant adenovirus is generated by joining the lacZ gene, which is a reporter gene for detecting the ability to activate the expression of the tTA gene, to the downstream side of the tetracycline-controllable tetracycline promoter (i.e., the Tet promoter; see M. Gossen et al., Proceedings of National Academy of Science, USA, Vol. 89, pp. 5547-5551, 1992). Then, gene transduction is performed by cotransfecting animal cells with these two types of adenoviruses. In this procedure, two types of recombinant adenoviruses are generated, one (control) carrying tTA alone (the same system as that of Bujard et al.) and the other carrying NtTA comprising tTA and a nuclear localization signal added thereto. These systems are comparatively examined for the ability to suppress gene expression in the presence of tetracycline and the ability to induce gene expres-

sion in the absence of tetracycline. As a result, it has been found that, in the absence of tetracycline, the degree of expression of the lacZ gene is about 30% in the tTA system, but 80% or greater in the NtTA system. Thus, the gene expression is enhanced in the NtTA system by a factor of 2.7 or greater.

On the other hand, it has also been found that, in the presence of tetracycline, the gene expression is completely suppressed in the NtTA system, whereas only an about 50% suppression of the gene expression is observed in the tTA system. By going through the above-described procedures to confirm the action of the respective recombinant genes, those skilled in the art will gain a better understanding of the present invention.

Furthermore, the present invention also provides the use of the above-described virus in combination with other viruses. For example, an adenovirus (hereinafter referred to as the first virus) containing a gene into which an appropriate promoter and NtTA are integrated can be used in combination with a recombinant adenovirus (hereinafter referred to as the second virus) containing a gene into which the gag-pol gene participating in the replication of retroviruses is integrated downstream of the tetracycline-controllable tetracycline promoter (i.e., the Tet promoter) and a recombinant adenovirus (hereinafter referred to as the third virus) containing a gene into which a gene encoding the envelope protein of a retrovirus is integrated downstream of the Tet promoter. This combination provides different uses.

For example, a high-level expression system for retroviruses can be provided by infecting animal cells with the aforesaid group of viruses. In another example, a sample of particular animal cells is co-infected with the first, second and third viruses. On

the other hand, another sample thereof is co-infected with the first and second viruses. Then, the transduction efficiencies of the gene in both samples are detected. If the transduction efficiency of the gene in the latter sample is as high as (or slightly lower than) that in the former sample, it may be presumed that the tested cells have been infected with any retrovirus. Thus, the present invention makes it possible to determine whether animal cells are infected with a retrovirus.

The present invention is more fully explained with reference to the following examples.

Example 1

The plasmid pUHD15-1 (see M. Gossen et al., Proceedings of National Academy of Science, USA, Vol. 89, pp. 5547-5551, 1992) was cleaved with XhoI and HindIII to obtain a 2.3kb CMtTA fragment. After this fragment was made blunt-ended, it was inserted into the SwaI site of the plasmid pAxcw to construct the cosmid pAxCMtTA (the restriction enzyme SwaI was purchased from Boehringer Mannheim and all other restriction enzymes were purchased from New England Bio Labo unless otherwise noted). Then, according to the method of Saito et al. (S. Miyake et al., Proceedings of National Academy of Science, USA, Vol. 93, pp. 1320-1324, 1996), human 293 cells (ATCC CRL-1573) were transfected with the above cosmid and the DNA-TPC (terminal peptide complex) of human adenovirus 5 to generate a recombinant adenovirus (AxCMtTA) (see Fig. 1).

The tTA gene was excised as an about 1kb fragment by cleaving pUHD15-1 with XbaI, making the resulting linear DNA blunt-ended, and then cleaving it with BamHI. On the other hand, the plasmid pRx·nZpA was constructed by adding the synthesized XR30-PK sequence (---CC ATG GAT AAA GCT GAA TTT CTC GAA GCT CCT AAG AAG AAA CGT AAG GTA GAA GAT CCT AGG AAT TC---; see D.

Kalderon et al., Cell, Vol. 39, pp. 499-509, 1984) to the 5'-end of the lacZ sequence of the plasmid pRxLacZ [obtained from Dr. Wakimoto (Cancer Chemotherapy Center, Cancer Institute)] so as to construct the plasmid pRx·nZ, and then replacing the 3'-LTR fragment (obtained by cleaving the plasmid pRx·nZ with BamHI and HindIII) with the SV40 poly(A) sequence which was an about 470bp fragment obtained by cleaving the plasmid pUHD15-1 with BamHI and HindIII. Subsequently, the plasmid pRxNtTA was constructed by cleaving the plasmid pRx·nZpA with EcoRI, making the resulting linear DNA blunt-ended, and ligating the aforesaid tTA gene into the BamHI site thereof. In this plasmid, the nuclear localization signal (NLS) of XR30-PK was joined to the tTA gene so as to be in-frame with the N-terminus of the protein encoded by the tTA gene.

The plasmid pTetZ was constructed by cleaving the plasmid pRx·nZ [obtained from Dr. Wakimoto (as described above)] with XbaI and BamHI to obtain an about 3.1kb NLS-lacZ fragment, and inserting this fragment into the XbaI/BamHI sites of the plasmid pUHD10-3 (see M. Gossen et al., Proceedings of National Academy of Science, USA, Vol. 89, pp. 5547-5551, 1992). This pTetZ was cleaved with XhoI and HindIII, and then treated with T4 DNA polymerase (purchased from New England Bio Labo) to generate blunt ends. Then, according to the above-described method of Saito et al., the resulting about 4.1kb fragment was inserted into the SwaI site of pAxcw to generate a recombinant adenovirus (AxTetZ) having the lacZ gene (see Fig. 1).

The plasmid pTetNtTA was constructed by cleaving the plasmid pRxNtTA with NcoI and BamHI to obtain an about 1.1kb fragment, and inserting this fragment into the NcoI/BamHI sites of the plasmid pTetZ. The plasmid pCMNtTA was constructed by cleaving the plasmid pUHD15-1 with EcoRI, making the resulting linear DNA blunt-ended,

cleaving it with XhoI to obtain a 0.77kb CM promoter fragment, and cloning this fragment into the XhoI site of the previously constructed plasmid pTetNtTA which had been cleaved with KpnI and made blunt-ended. In order to generate a recombinant adenovirus (AxCMNtTA) for the expression of the CMNtTA gene, the CMNtTA gene was excised as a 2.3kb fragment by cleaving the plasmid pCMNtTA with XhoI and HindIII, treated with T4 DNA polymerase to generate blunt ends, and then cloned into the SwaI site of the cosmid pAxcw to construct the cosmid pAxCMNtTA. Thereafter, the recombinant adenovirus AxCMNtTA was generated according to the above-described method of Saito et al. (see Fig. 1).

HeLa cells were infected with the generated recombinant adenoviruses AxCMtTA and AxCMNtTA in the MOI range of 10 to 1250. Thus, it was found that, at a MOI of 250 and in the absence of tetracycline, the gene expression in the system using the NtTA-carrying adenovirus was about 2.7 times as high as that in the system using the tTA-carrying adenovirus. In the presence of tetracycline, the gene expression was completely suppressed in the system using the NtTA-carrying adenovirus, whereas only an about 50% suppression was observed in the system using the tTA-carrying adenovirus (see Fig. 2).

Example 2

In order to construct the plasmid pCANtTA, the plasmid pCAGGS (see H. Niwa et al., Gene, Vol. 108, pp. 193-200, 1991) was cleaved with SalI and HindIII. The resulting fragment was made blunt-ended and combined with the ClaI linker to construct the plasmid pCAcc. After this plasmid pCAcc was cleaved with EcoRI and made blunt-ended, an about 1.5kb fragment obtained by cleaving the plasmid pTetNtTA with KpnI and HindIII and making the resulting DNA fragments blunt-ended was inserted thereinto. Then, according to the above-des-

cribed method of Saito et al., an about 2.7kb fragment obtained by cleaving the plasmid pCANtTA with ClaI was inserted into the ClaI site of the plasmid pAxcw to generate a recombinant adenovirus (AxCANtTA) (see Fig. 1).

An about 1kb tTA fragment was obtained by cleaving the plasmid pUHD15-1 with XbaI, making the resulting linear DNA blunt-ended, and then cleaving it with BamHI. Then, the plasmid pRxNtTA was constructed by cleaving the plasmid pRx·nZpA constructed in Example 1 with EcoRI, making the resulting linear DNA blunt-ended, and inserting the aforesaid tTA gene into the BamHI site thereof. Then, according to the above-described method of Saito et al., an about 3.6kb fragment obtained by cleaving the plasmid pRxNtTA with XhoI and HindIII and making the resulting DNA fragments blunt-ended was inserted into the SwaI site of the plasmid pAxcw to generate a recombinant adenovirus (AxRxNtTA) (see Fig. 1).

An about 1.1kb fragment obtained by cleaving the plasmid pRxNtTA with NcoI and BamHI was inserted into the NcoI/BamHI sites of the plasmid pTetZ constructed in Example 1 to generate pTetNtTA. Then, according to the above-described method of Saito et al., an about 2.1kb fragment obtained by cleaving the pTetNtTA with XhoI and HindIII was inserted into the SwaI site of pAxcw to generate a recombinant adenovirus (AxTetNtTA) (see Fig. 1).

HeLa cells were infected with the generated recombinant adenoviruses AxCANtTA, AxCMNtTA, AxRxNtTA and AxTetNtTA at a MOI of 250. Thus, in the absence of tetracycline, the degree of gene expression was 85.5% in the system using the CA promoter, 82.7% in the system using the CM promoter, 82.4% in the system using the Rx promoter, and 39.9% in the system using the Tet promoter. In the presence of tetracycline, the gene ex-

pression was completely suppressed in all of the four systems. These results and the results of Example 1 are summarized in Table 1, indicating that the CA, CM and Rx promoters exhibited almost equal gene expression activities, but the gene expression activity exhibited by the Tet promoter was only about a half. However, all of the systems using NtTA exhibited higher gene expression activities than the system using CMtTA (see Fig. 3).

Table 1

Comparison of the Expression Efficiencies of the Introduced Gene by Use of Nuclear Localization Signal (NLS) and Various Promoters

Gene expression system	AxCANtTA	AxCMNtTA	AxRxNtTA	AxTetNtTA	AxCMtTA
Expression efficiency (%)	85.5	82.7	82.4	39.9	29.2

In the presence of tetracycline, the gene expression in the cells into which the gene had been introduced by using each of the generated adenoviruses was completely suppressed at tetracycline concentrations of 1 μg/ml or greater in the systems using NtTA. In the tTA system, however, the gene expression was not completely suppressed even at a tetracycline concentration of 100 μg/ml. Moreover, it was also found that, in the absence of tetracycline, the expression efficiency of the introduced gene was 80% or greater in the CMNtTA, CANtTA and RxNtTA systems, but only about 25% in the CMtTA system (see Fig 4).

Example 3

HeLa cells were co-infected with the recombinant adenoviruses AxCMtTA or AxCMNtTA and AxTetZ generated in Example 1, cultured in the presence or absence of tetracycline, and then subjected to β-Gal staining. As a result, in the absence of tetracycline, only about 30% of the total cells were stained in the tTA system (AxCMtTA and AxTetZ), but approximately 100% of the total cells were stained in the NtTA system (AxCMNtTA and AxTetZ). In the presence of tetracycline, no cell was stained in the NtTA system, indicating that the expression of β-Gal was completely suppressed. However, some (15%) of the cells were stained in the tTA system, indicating that the expression of β-Gal was suppressed by only about 50% (see Figs. 5 and 6).

Example 4

The plasmid pSKII+VSVG was constructed by using an about 1.6kb fragment obtained by cleaving the plasmid pLGRNL (see N. Emi et al., Journal of Virology, Vol. 65, pp. 1202-1207, 1991) with BamHI. The plasmid pTetVSVG was constructed by cleaving the plasmid pSKII+VSVG with BamHI and inserting the resulting about 1.6kb fragment into the BamHI site of the plasmid pUHD10-3. Then, according to the method of Saito et al., an about 2.4kb fragment obtained by cleaving the plasmid pTetVSVG with XhoI and HindIII and making the resulting DNA fragment blunt-ended was inserted into the SwaI site of the plasmid pAxcw to generate a recombinant adenovirus (AxTetVSVG) (see Fig. 1).

HeLa cells were co-infected with the generated recombinant adenoviruses AxTetVSVG and AxRxNtTA, and then subjected to immunohistochemical staining with a monoclonal anti-VSVG antibody (clone P5D4, Sigma #V5507). Thus, the expression of the VSVG gene product was observed in approximately 100% of the cells. In contrast, when HeLa cells were infected with any one of

the aforesaid recombinant adenoviruses, the expression of the VSVG gene product was not observed in either case.

Example 5

In order to monitor the production efficiency of a retroviral vector, human cultured cells such as A375, U373, T98G and A172 were infected with the retroviral vector MFGlacZ expressing the reporter gene lacZ (see Dranoff et al., Proceedings of National Academy of Science, USA, Vol. 90, pp. 3539-3543, 1993). Thus, there were obtained gene-transduced cell lines such as A375/Z, U373/Z, T98G/Z and A172/Z. The transduction efficiency of the lacZ gene was evaluated by a combination of detection of lacZ expression with a flow cytometer and detection by X-gal staining. For all of A375, U373, A172 and T98G, the lacZ gene-transduced cell lines were obtained with an efficiency of 50 to 100%.

The 5307bp gag-pol gene extending from nt563 (HindIII) to nt5870 (ScaI) was excised by cleaving MoMLV (see Schnich et al., Nature, Vol. 293, pp. 543-548, 1981) with HindIII and ScaI. The Tet promoter was excised by cleaving pTetZ with XhoI, making the resulting linear DNA blunt-ended, and then cleaving it with EcoRI. Then, the plasmid pTetGP was constructed by cloning the gag-pol gene and the Tet promoter into the SpeI and BglII sites of pCAcc. (In fact, however, pTetGP was constructed by a plurality of steps in which the gag-pol of MoMLV was fragmented, these fragments were subcloned into pBluescript SKII and further subcloned into pCAcc to construct pCA·GP, and its CA promoter was replaced by the Tet promoter.) The cosmid pAxTetGP was constructed by cloning into the ClaI site of pTetGP. Moreover, 293 cells were co-infected with the cosmid pAxTetGP and Ad5·DNA-TPC to generate a recombinant adenovirus (AxTetGP).

The previously prepared A375/Z, T98G/Z, A172/Z

and U373/Z cells were co-infected with all or some of the generated recombinant adenoviruses AxTetVSVG, AxTetGP and AxCANtTA. After 2 days, the culture supernatants considered to contain a retrovirus were recovered. Using each of these culture supernatants considered to contain a retrovirus, monitor cells (e.g., NIH 3T3 cells) were infected therewith in the presence of 8 μg/ml Polybrene (Sigma), and subjected to X-Gal staining after 2 days. Then, the efficiency of infection with the retrovirus was calculated. The results thus obtained are summarized in Table 2. These results reveal that pseudotyped retroviruses having the lacZ gene integrated thereinto can be recovered from the A375/Z, T98/Z and U373/Z cells at a high titer of 10^5 to 10^6 pfu/ml. On the other hand, the retrovirus production capacity of the A172/Z cells was low. In the A375/Z cells, the retrovirus was produced without simultaneous infection with the adenovirus AxTetVSVG. This indicates that A375 cells have therein a gene for env-like protein which is required for the replication of retroviruses.

Table 2

Transduction Efficiency of
VSVG-pseudotyped Retroviral Gene

	Gene transduction efficiency (%)			
	A375/Z	T98/Z	A172/Z	U373/Z
MOCK	0	0	0	0
TetVSVG alone	0	0	0	0
TetGP alone	0	0	0	0
CANtTA alone	0	0	0	0
TetVSVG+TetGP	0	0	2/0*	0
TetVSVG+CANtTA	3	0	0	0
TetGP+CANtTA	42.0±6.66	0.83±0.983	1.5±0.84	2.0±0.89
CANtTA+TetVSVG+TetGP	71.3±5.92	38.7±5.85	5.3±2.80	46.8±6.94

* Gene expression was observed in one of two plates.

CLAIMS

1. A recombinant gene comprising a nuclear localization signal (NLS) gene and the tetracycline transactivator (tTA) gene.

2. A recombinant gene as claimed in claim 1 wherein the NLS gene and the tTA gene are integrated in-frame.

3. A recombinant gene as claimed in claim 1 wherein the NLS gene is derived from the large T protein of SV40 virus.

4. A recombinant gene as claimed in any one of claims 1 to 3 which further comprises a promoter gene.

5. A recombinant gene as claimed in claims 4 wherein the promoter gene is selected from the group consisting of the CM promoter of cytomegalovirus, the Rx promoter comprising the enhancer of cytomegalovirus joined to the promoter of Moloney murine leukemia virus, the CA promoter comprising the enhancer of cytomegalovirus joined to the promoter of β-actin, and the Tet promoter of the tetracycline gene.

6. A recombinant gene as claimed in claims 5 which further comprises a gene encoding a foreign protein.

7. A recombinant gene as claimed in claims 6 wherein the foreign protein comprises at least one member selected from the group consisting of viral envelope proteins, hormones, cytokines, and the receptors for hormones and cytokines.

8. A plasmid carrying a recombinant gene as claimed in any one of claims 1 to 7.

9. A virus containing a plasmid as claimed in claim 8.

10. A virus as claimed in claim 9 wherein the host virus is an adenovirus.

11. A virus as claimed in claim 10 wherein, in the recombinant gene contained therein, the NLS and tTA

genes integrated in-frame are located downstream of the promoter.

12. An animal cell transfected with a virus as claimed in any one of claims 9 to 11.

13. An animal cell as claimed in claim 12 wherein the transfected cell is a mammalian tumor cell.

14. A group of viruses comprising a virus as claimed in claim 11, combined with a recombinant adenovirus containing a recombinant gene into which the gag-pol gene participating in the replication of retroviruses is integrated downstream of the tetracycline-controllable tetracycline promoter (the Tet promoter), and a recombinant adenovirus containing a recombinant gene into which a gene encoding a retroviral envelope protein is integrated downstream of the Tet promoter.

15. A group of viruses as claimed in claim 14 wherein the retroviral envelope protein is the membrane-penetrating protein of vesicular stomatitis virus.

16. An animal cell co-infected with a group of viruses as claimed in claim 14 or 15.

17. A method for producing a retrovirus which comprises co-infecting an animal cell with a group of viruses as claimed in claim 14 or 15, and growing the resulting animal cell.

18. A method for detecting the infection of an animal cell with a retrovirus which comprises co-infecting animal cells suspected of infection with a retrovirus, with one or more viruses included in a group of viruses as claimed in claim 14 or 15, and detecting a tendency exhibited by the retrovirus.

FIG. 1

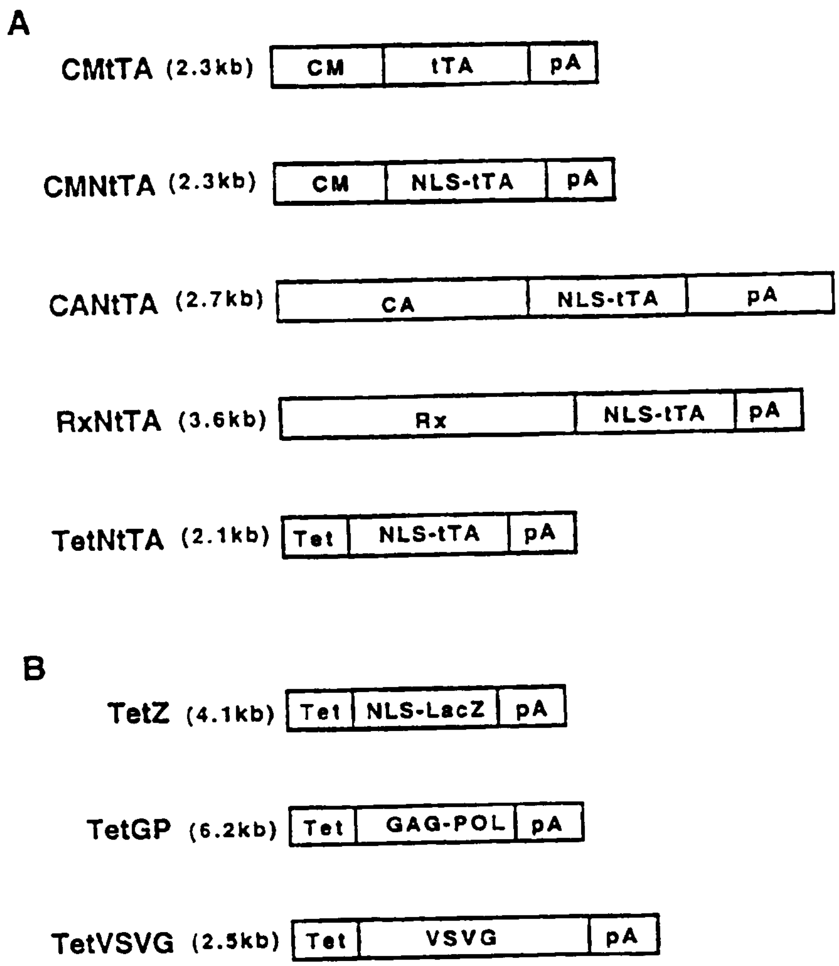

A
CMtTA (2.3kb)
CM
tTA
pA
CMNtTA (2.3kb)
CM
NLS-tTA
pA
CANtTA (2.7kb)
CA
NLS-tTA
pA
RxNtTA (3.6kb)
Rx
NLS-tTA
pA
TetNtTA (2.1kb)
Tet
NLS-tTA
pA
B
TetZ (4.1kb)
Tet
NLS-LacZ
pA
TetGP (6.2kb)
Tet
GAG-POL
pA
TetVSVG (2.5kb)
Tet
VSVG
pA

FIG. 2

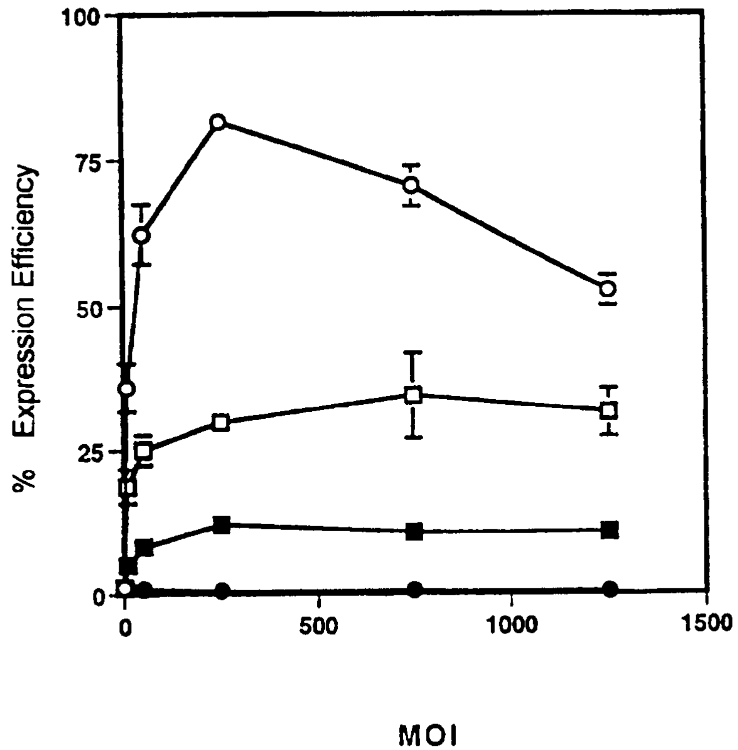


FIG. 3

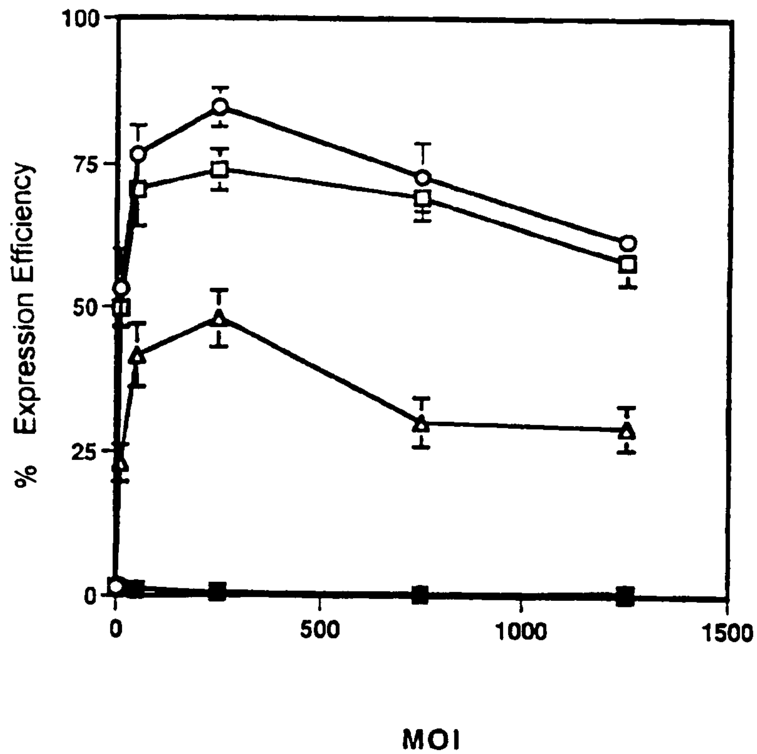


FIG. 4

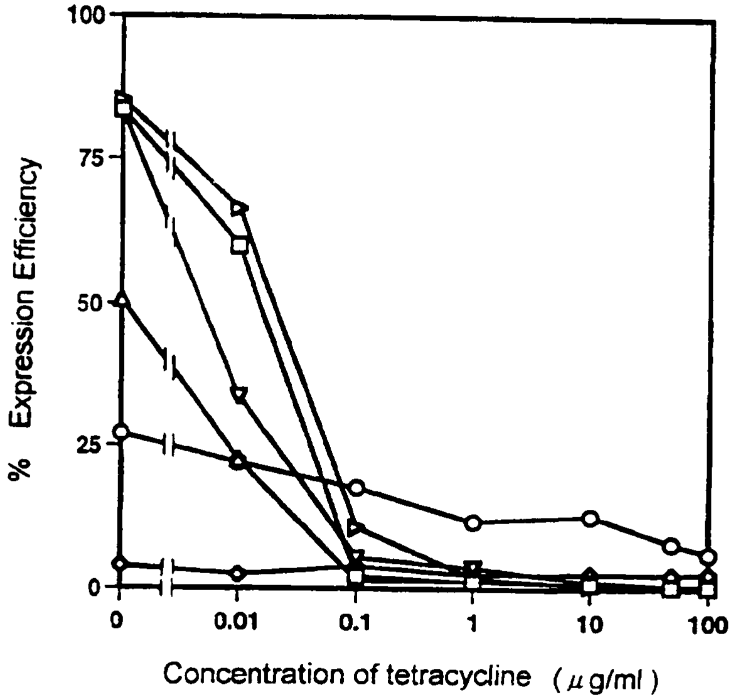

100
75
50
25
0
% Expression Efficiency
0
0.01
0.1
1
10
100
Concentration of tetracycline (μg/ml)

FIG. 5

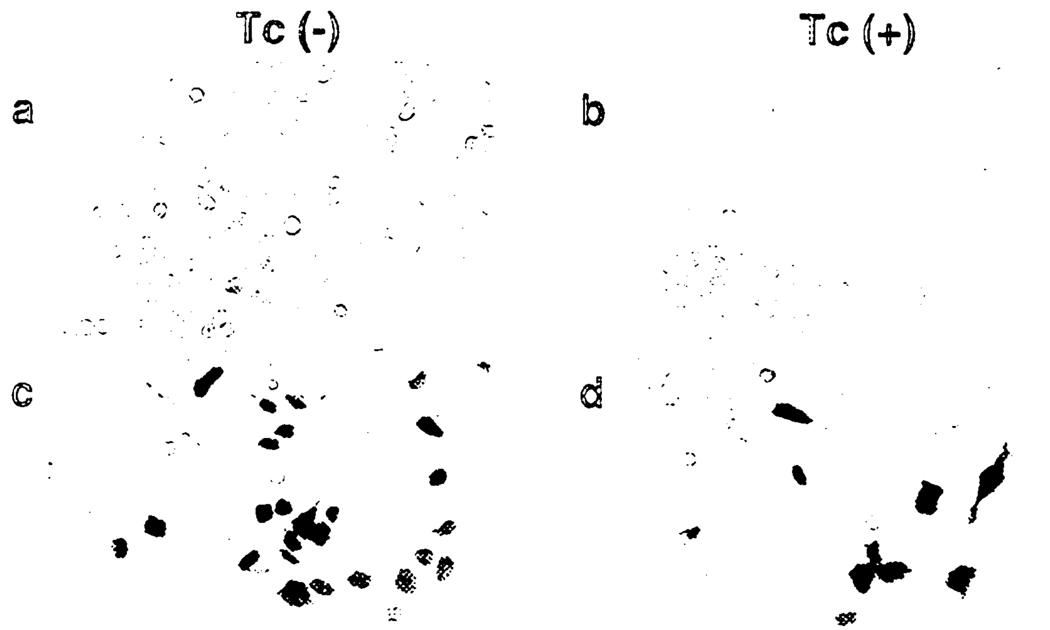
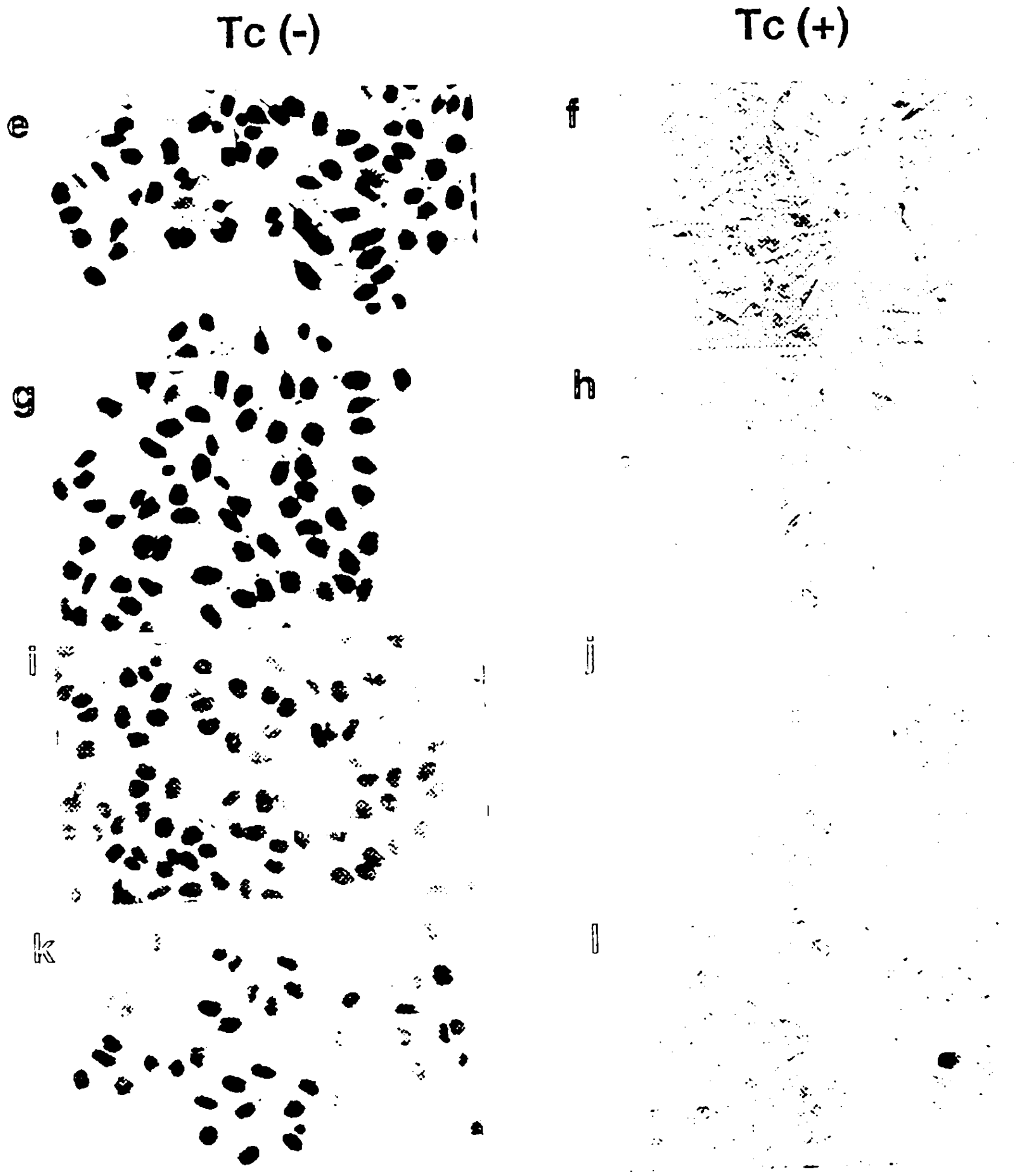


FIG. 6



INTERNATIONAL SEARCH REPORT

International Application No
PCT/JP 97/03109

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 //C07K14/245

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 97 35992 A (VICAL INC.) 2 October 1997 see abstract see page 3, line 5 - page 4, line 21 see page 15, line 27 - line 34 see claims ---	1-6,8
Y	SONEOKA Y ET AL: "A TRANSIENT THREE-PLASMID EXPRESSION SYSTEM FOR THE PRODUCTION OF HIGH TITER RETROVIRAL VECTORS" NUCLEIC ACIDS RESEARCH, vol. 23, no. 4, 25 February 1995, pages 628-633, XP000569533	5-7,14
A	see abstract see page 630, column 1, paragraph 2 see page 630, column 2 - page 631, column 2 see page 633, column 1, paragraph 2 ---	17,18

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
18 December 1997	11.02.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

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Intern. ...al Application No
PCT/JP 97/03109

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GOSSEN M ET AL: "TIGHT CONTROL OF GENE EXPRESSION IN MAMMALIAN CELLS BY TETRACYCLINE-RESPONSIVE PROMOTERS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 12, 15 June 1992, pages 5547-5551, XP000564458 cited in the application see the whole document ---	1-8, 12-14
Y	WO 95 19368 A (ALEXION PHARMA INC) 20 July 1995 see abstract see claims see page 5, line 9 - page 8, line 13 see page 9, line 14 - page 15, line 19 ---	1-6,8, 12,13
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X,P	YOSHIDA Y. ET AL.: "Highly efficient vsvg-pseudotyped retroviral packaging system through adenovirus-mediated inducible gene transduction - recombinant adenovirus-mediated vesicular-stomatitis virus-G and mouse Moloney leukemia virus gag and pol gene transfer to glioma for cancer gene therapy" CANCER GENE THERAPY, vol. 3, no. 6 Conference suppl., 14 November 1996, pages s21-s22, XP002050699 see the whole document ---	1-18
A	WO 94 29440 A (UNIV CALIFORNIA) 22 December 1994 see the whole document -----	14-16

1

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Information on patent family members

Intern. ial Application No
PCT/JP 97/03109

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